CHAPTER 1

INTRODUCTION
**Bacillus thuringiensis toxin proteins as biopesticide:**

Every year millions of dollars is lost all over the world due to insect damages incurred on crop produces. This has been a major concern for the crop growers. In order to prevent such a loss, application of chemical pesticides has been the usual measure adopted by the farmers, despite the demerits of such application; such as high price, environmental persistence and adverse effects on non-target organisms including human. Consequently, the cost of insect control through the use of chemical pesticides has been high in terms of both economy and environment. Search for alternative, yet cost effective, environmentally safe and sustainable insect control agents has identified Bacillus thuringiensis toxin proteins as the most promising and powerful class of biopesticide. In fact, for decades, farmers have been using Bt toxin preparations in their field for crop protection. Sensing the importance of Bt toxins, many agricultural companies came forward to produce commercial Bt toxin sprays (such as Thuricide™ and Dipel™).

*Bacillus thuringiensis* is a gram positive, soil borne, spore forming bacterium and it produces insecticidal crystal proteins (ICPs) of ~ 130 kDa molecular mass during sporulation stage (1). ICPs are protoxins, dissolved in high alkaline pH. Upon ingestion by the larvae they are digested by specific proteases present in insect midgut releasing the N-terminal active toxin peptide (~65 kDa). This active peptide binds to specific receptor proteins present on the insect midgut epithelial cell membrane. It inserts itself to the membrane and forms ion channels leading to
colloidal osmotic lysis of the cells (2). These proteins are diverse in their insect specificity (1). Each kind of toxin protein is active in only one or a few insect species. Insect specificity is determined by the toxin receptor interaction (3). Bt toxins have no detrimental effects on non-target organisms, mainly because of their high specificity (4). So far, more than 130 different Bt crystal proteins toxic to different insects, nematodes, and protozoans have been reported and are classified mainly based on their spectrum of activity and amino acid homology (http://www.epunix.biols.susx.ac.uk/Bt.index (1,5).

1.1 The toxin molecule:

Informations on toxin structure have come mainly from structural and mutational studies. Insight on to the 3-D structures of CryIa and CryIIA has been developed from x-ray crystallographic studies (6,7). Both CryIa and CryIIA are structurally similar, although they differ in their amino acid sequences and their host specificity. Both of them form globular proteins and are very stable at wide ranges of pH. Both proteins contain three functionally distinct domains, which are connected to each other by hydrogen as well as ionic interactions. There are many salt bridges between domain I and domain III that make the protein stable over a wide range of pH. It was also found that at these pH ranges there are no or little conformational changes in their structure (7). These informations along with mutational studies of a number of toxin proteins elucidated the primary structure responsible for pore formation and specificity for toxicity (8,9,10). In CryIIA, the N-terminal 290 residues form a bundle of seven helices (α 1-7) forming the domain I (6). The domain I is rich in hydrophobic and amphipathic amino acids. It has been shown that the
helices of domain I are responsible for the formation of voltage dependent ion channels in the lipid bilayer of epithelial plasma membrane of the insect midgut (11,12,13). The CryIAa toxin having 90% homology with CryIAc, the domain I extend from residues 33 to 253 (7). Residues from 291 to 500 consist of three antiparallel β-sheets packed around a hydrophobic core with a triangular cross section known as domain II. Mutational studies implicate this region to binding on specific receptors present on the midgut epithelium (15,16,17,18,22). Very little homologies are found in this region among different types of toxins. The domain III starts from 501 and ends with 644 amino acids. It consists of sandwich of two antiparallel β-sheets. Recent experiments using hybrid toxins indicate direct involvement of this region in receptor binding (15,19,20,21). Four intermolecular salt bridges between domain I and III make the toxin insoluble until exposed to the extreme pH in the insect midgut (7). This structure is more or less considered to be common for all the Cry toxins as revealed from mutational studies.

1.2. Mode of action of Bt toxins:

The common mechanism of midgut epithelial cell lysis by the toxin molecules is believed to be by the formation of lytic pores of 10-20 Å diameter in the insect midgut membrane (6). Mutation studies attribute domain I to pore formation (22,23). The active toxin binds to the specific receptor proteins present on the epithelial plasma membrane of the insect midgut. This triggers a conformational change in the toxin, helping the insertion of domain I into the epithelial cell membrane (26). The initial binding is believed to be reversible (27). After binding, the domain I of the bound peptide insert itself to the plasma bilayer forming channels that cause rapid ion
movement from the epithelial cells leading to osmolysis and the binding becomes irreversible (27). Both binding and pore formation are equally important in toxicity. The specificity of the delta endotoxins is determined by the affinity with which it binds to the receptor proteins present in the insect midgut membrane. These receptors are glycoproteins as observed in certain cases (35, 36, 37, 38). Past studies have identified that residues 365 to 371 of CrylAa are essential for binding to membrane midgut receptors in *Bombyx mori* (39). In three-dimensional structure, this segment forms a flexible and highly mobile loop (7). The loop structure of CrylAa is structurally dissimilar to the loop structure of CryllIA (7) and this could be one of the reasons for their differences in specificity. CrylAa is Lepidoptera specific and CryllIA being Coleoptera specific. The CrylIA toxins are sensitive to insects belonging to Lepidoptera and Diptera. The dual specificity of CrylIA for Lepidoptera and Diptera is determined by residues 307-382 (13). On the other hand, the specificity-determining region of CrylAe to lepidoptera is located to three different regions of domain II (14). Mutant proteins produced by reciprocal recombination between CrylAa and CrylAe show differences in their specificity towards *Heliothis virescens* and *Trichoplusia ni* (41). These observations do sufficiently indicate that the interaction and involvement of both domain II and domain III play functional roles in receptor recognition.

1.3. Background of the present study

Keeping in view of the *Bt* toxin peptide as an entomocidal agent, following merits of the *Bt* toxin over other insecticides are being pointed out.
a) Bt toxin is a biopesticide and environmentally safe unlike other chemical pesticides.

b) Bt toxin is sensitive to specific target insects, hence, it is likely not to be toxic to the beneficial and non-target organisms.

c) Very low dose of the toxin is effective against the target insect, hence, cost of production of it is economic.

d) Basic knowledge on the structural and functional properties of the toxin molecule as an entomocidal agent is available. These informations open up scopes for further manipulation of the entomocidal property to suit to particular requirement.

e) Diverse forms of Bt toxin with wide spectrum of specificity towards different insects and other invertebrates exist in nature. The versatility of this entomocidal agent opens up new possibilities for its use as a potential component for insect control management strategies.

Nevertheless, there are still some outstanding disadvantages that have become known in the use of Bt toxin in insect control. They are:

a) A single toxin is specific for one or few insect pests. When a crop is infested with large number of pests, like in tropical crops, a single toxin may not be sufficient for overall protection of the crop yield.

b) Insects develop resistance towards Bt toxins under selection pressure. By inbreeding, these insects may grow in large number in a short time.
a) Stability of Bt toxins is poor against sun rays. When used as spray, it cannot be effective against insects that avoid feeding on outer surface of the crop plant. The toxin can also be washed away during rain.

In order to counter these unwanted possibilities transgenic approach for expression of Bt genes in planta has appeared as a potential approach for insect control in agricultural biotechnology.

With the recent progress made in genetic engineering of plants and the success already attained in production of transgenic plants with Bt genes expressing the toxin peptide in plant, transgenic strategy for development of protection against insect has been a potential tool. This laboratory is engaged in exploring possibilities for the use of Bt toxin genes to control insect pests of major crops of India. The present study has been addressed to develop ways to counter the following issues that determine the efficacy of any single Bt toxin molecule as genetical elements to prevent insect damage to crop plants. Based on the initial success attained by this laboratory in transforming a truncated native crylAc gene in chickpea, (Cicer arietinum L.) (50), a major legume crop of India, the present program of study was initiated in order to improve upon the potentiality of the Bt chickpea plants to control more effectively the damage caused by podborers (Helicoverpa armigera. Hub.) The transgenic plants developed through the use of a native bacterial crylAc gene showed moderate levels of protection against the insect pest, which was found to be inadequate. In order to fulfill the requirement, following aspects figured as target issues, in the present study.

1. Reconstruction of the bacterial crylAc gene to ensure high level of expression in plant.
b) Answers were sought to the following questions: i) Does crylAc gene offer scope for modification of its structure, so that it's functional properties can be altered to broaden its toxic potential to other lepidopteran insect crop pests? ii) Furthermore, can CrylAc molecule be altered such that novel binding proteins in insects could be identified, which can in turn result into mortality of insect pests that are resistant to native CrylAc molecule?

In order to fulfill the first target, a research program was initiated to produce insect resistant transgenic chickpea plants. Chickpea is an important legume crop and its seeds are rich in nutritive value. Worldwide, it is accepted as a major source of vegetable protein for human as well as animal consumption. India is the largest producer of chickpea, contributing over 70% of the total world production (51). Loss of more than 30% of this crop production is inflicted due to the damages caused by pod borers (*Helicoverpa armegera* Hub.), a major lepidopteran pest. The larvae preferentially feed on developing seeds within the pod resulting serious yield loss. Genes conferring resistance to pod borer damages are not available in the germplasms of *Cicer* sp. Therefore, the only choice left with the farmer has been the use of chemical pesticides to protect the crop yield against insect infestation. Thus, generation of transgenic insect resistant chickpea plants carries distinct possibility to bear significance.

To answer the second objective posed, attempts have been directed to test for the possibility for modification of the CrylAc toxin in order to alter its mortality mechanism. This, we set out to carry through domain swapping principle. We targeted CryllA toxin as the source for a variant domain. CryllA is known to be toxic
to both Lepidoptera and Diptera (114), and is phylogenetically quite distinctly apart from CryIAc toxin. On the basis of amino acid sequence alignment, it was estimated through this study that CryIIA share only 21.87 % of identity with CryIAc toxin. Although an earlier estimation with regard to similarities between CryI type of toxins with that of CryIIA toxin had been estimated to be 37% (115). Past study has indicated that CryIIA toxin has a unique mode of insect midgut receptor binding activity indicating a different mortality mechanism (14). We set out to make use of these informations for generating hybrid toxins by swapping the functional domains between these two proteins.

1.4.Insect resistant transgenic crop plants: a brief status account

Since gene transfer in plants became a reality, it became apparent that genetic engineering methods would provide solutions to many problems associated with crop improvement. Indeed, cloning of genes that code for entomocidal endotoxins by various strains of Bacillus thuringiensis and transfer of some of such genes to plants to develop insect resistant transgenic plant have been one of the first significant successes of plant biotechnology. Over the past few years, production of many insect resistant crop plants and their utilization in the field condition have been reported (50,77,78,79). Many plants, especially Cotton, Maize, Potato, and Tomato have been commercialized in different parts of the world (http://www.monsanto.com/ag/articles/PlantBiotech). Such transgenic plants have demonstrated their advantages over application of Bt toxin formulations. Constitutive expression of Bt gene in plant ensures its availability to insects harboring crop plants while feeding on plant tissues. Transgenic plants are especially effective on the
insects that bore inside the tissue, which otherwise are inaccessible to sprays. Lepidopteran pests like rice stem borer, cotton bollworm, chickpea pod borer, and maize corn borer represent for this type of insect pests.

In the early transgenic studies for insect resistant plant development, both full length and truncated cry genes were introduced into tobacco and tomato (52,53). However, only gene constructs that contained truncated gene (53,54), yielded plants with some measure of protection against insect damage. However, in such cases the transgenic plants showed inadequate expression of the transferred gene both at transcriptional and translational level, despite the use of strong plant promoters, like MAS (53) and CaMV 35 S (54). Simultaneous observations relating to Bt genes that are in general rich in AT content led to the speculation that the AT richness may play a negative role in their expression in plant system. The Bt genes are characterised by 65% AT content, whereas plant genes usually contain 45-55% (monocot-45%, dicot - 55%) (66). Further studies relating to the characteristics of the transcripts, their stability, and their translatability, led to the realization that the transcripts of native Bt genes in plants are unstable and are poorly translated (55,57,58,59,60). Bt gene sequences show presence of regions similar to plant mRNA processing signals. This could probably be the reason for the presence of truncated, polyadenylated and irregularly spliced transcripts in plant tissues (55,57). Moreover, half-life of the Bt gene transcripts in plants has also been observed to be low, indicating instability of the transcripts (58,59). All these factors contributed towards low expression of Bt genes in plant.
Attempts to increase the expression of truncated Bt genes by eliminating the negative regulatory elements yielded enhanced expression. More than 10-fold expression was obtained with the help of partially modified genes (77,78). Partial modification, as well as modification of the 5' one third of the coding region or 3' two third of the coding region did not increase expression to the level that a fully modified gene is capable of (77,78,80). This is caused due to the presence of negative regulatory sequences throughout the coding region. Thus, for the required higher expression in plants, complete elimination of these sequences is necessary. This could be achieved only by reconstructing the gene, completely. Based on the above understanding, crylAc, crylAb, crylC, crylIA and crylIIA genes amongst some more have been reconstructed and transferred to cotton, rice, maize and potato, besides other crop plants (28,77,78,79,81). In certain cases, 100-fold enhancement of expression could be realised than that of the native genes. Parallely, efforts have also been made to express the native Bt gene in chloroplast via chloroplast transformation (82). The logic behind such an attempt has been that since the genetic apparatus of a chloroplast is prokaryotic in nature, expression of the bacterial Bt gene should be a routine process. 500-fold excess expression of Bt gene could also be observed in chloroplast transformed tobacco plants. However, the bottom line of this success has been that chloroplast transformation as a technique itself has been the bottleneck for expanding the use of this technique, as beyond tobacco in no other plants, success has yet been achieved.
1.5. Insect resistance against Bt toxin

A serious challenge against the virtues of Bt insecticide has come from the rapid emergence of resistant insect pests against the toxin. Insects tend to adapt fast when exposed to chemical as well as biological pesticides and gain resistance within a short time. Many insect strains have been observed to develop resistance in the laboratory selection as well as in field conditions (30,32,33,84,86). Development of resistant insects can in an insect protection strategy, jeopardize the gains of transgenic Bt plants for insect control. It is believed that the genes conferring resistance to insects are present in the natural population of insects. In presence of the Bt plants, a selective pressure is generated for the emergence of resistant insects, as sensitive ones are eliminated. Mating between surviving resistant insects can then generate fairly large number of resistant insect population within a short time to cause damage to the Bt crop plants. To combat such an eventuality, different strategies have been proposed (100,101,102,103).

In laboratory selected strain of Plodia interpunctella resistant to Bt toxins, it was shown that resistance is related to a change in the affinity to the membrane receptor (31,33,97). In another study on field population of Plutella xylostella resistant to CryIAb, it was shown that the receptors lost the capacity to bind to CryIAb (85). It is assumed that the toxin peptide acts in two steps: specific binding to receptor proteins followed by membrane disruption. This means, only binding would not ensure toxicity. It has been indicated that some toxins bind to receptors without being toxic. Similarly, crystal proteins can't be toxic, if it is not able to bind to the receptor (89). Studies on Plutella xylostella resistant to CryIAc shows that binding of
the toxin to the brush border membrane receptors is not sufficient for toxicity (99). Studies on CryIA resistant and susceptible strain of diamondback moth *Plutella xylostella*, using different Cry I toxins and their hybrids point to the altered interactions of domain II and the receptor of resistant strain (99).

1.6. Strategies for insect resistance management

Different strategies have been put forward and some have been adopted in order to delay or prevent adaptation in the insect pest (100, 101, 102, 103). Use of *Bt* insecticidal toxin spray and/or transgenic plant is designed to represent for an important component of a potential and most useful integrated pest management (IPM) strategy. For the use of Bt toxins the following strategies have been proposed.

1. Diversification of mortality sources.
2. Reduction of selection pressure.
3. Use of susceptible insects as refuges.

Single, multiple and/or chimeric toxins can be used simultaneously or at different time. Similarly, the protein production can be regulated in transgenic plants by the use of different promoters. By this way, a required level of the toxin (high, low, or moderate) can be obtained.

Based on the fact that the mortality mechanism of a toxin to any target insect is specific, a toxin with different mechanism to the insect can be utilised for countering damages caused by resistant insects. Other proteins, apart from crystal proteins of *Bacillus thuringiensis* could also be efficient insecticidal agents (second-generation toxins). Vegetative insecticidal proteins (VIPs) of *Bacillus thuringiensis* are novel classes of toxin with toxicity equal to Cry proteins. Protease inhibitors from
soyabean and cowpea plant lectins, cholesterol oxidase and polyphenol oxidase are all promising biopesticides (104). However, availability of an equally toxic protein to any target insect seems not to be readily available. Thus, through this study an approach to generate chimeric toxins, by combining asymmetrical domains of the cryIIA toxin molecule in the background of cryIAc toxin molecule has been undertaken. Through similar approach in the past, a number of novel toxins have been developed by recombining CryIC and CryIE (98). Some of the recombinant proteins showed broader activity and could bind to different receptors than the parent toxins. It has been also strongly argued in these cases that such proteins can be of use in the wake of resistance development among insect pests.
Objectives of the present study:

The objectives of the present study, thus, have been fixed as:

a) Development of a suitably reconstructed \textit{cryLa} gene to ensure high expression in plant.

b) Generation of transgenic chickpea lines with the reconstructed \textit{cryLa} gene in order to protect the plants against the damages caused by the pod borers.

c) Modification of the \textit{cryLa} toxin molecule through domain swapping approach to generate chimeric \textit{cryLa} toxin molecule with domains of \textit{cryIIA}. It is believed that elements of such a diverse toxin molecule would carry greater possibilities for generation of toxin molecules with novel entomocidal properties.

The following text gives an account of the results that have been generated along the lines, as indicated above.